ROLE OF PEGYLATION ON THE CIRCULATORY STABILITY AND IMMUNOGENICITY OF RECOMBINANT CHOLINESTERASES

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ABSTRACT

Previous studies in rodents and non human primates demonstrated that pretreatment of animals with cholinesterases (ChEs), both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), could provide significant protection against behavioral and lethal effects of nerve agent intoxication. Currently, human (Hu) serum BChE is under development as a medical countermeasure against organophosphate chemical warfare agent toxicity. Similarly, methods for producing gram quantities of recombinant (r)Hu BChE and its development as a bioscavenger are also being pursued. In this regard, we examined the pharmacokinetic and immunologic properties of rHu ChEs and found that they displayed a relatively short half-life and enhanced immunogenicity in mice. To improve their circulatory stability, we modified recombinant (r)Hu ChEs by polyethylene glycosylation (PEGylation) and evaluated PEG modified proteins for size modification by SDS-PAGE, any changes in catalytic and inhibitory parameters, alterations in pharmacokinetic behavior, and their immunogenic activity. PEGylation of rHu ChEs with 20 kDa PEG polymer (PEG-rHu ChEs) had no deleterious effects on the catalytic properties of the enzymes. When administered into mice, a significant improvement in the circulatory stability was observed for PEG-rHu ChEs. Pharmacokinetic parameters for rHu ChEs were similar or close to those for native ChEs. However, a second injection of PEG-rHu ChEs administered 28 days later cleared rapidly from the circulation of mice as compared to the first injection. The poor circulatory stability observed for the second injection of PEG-rHu ChEs coincided with the presence of circulating anti-ChE IgG levels in mice. In conclusion, although PEGylation represents a suitable simple strategy to improve the circulatory stability of rHu ChEs, it may not be a suitable approach to eliminate the immunogenicity of these enzymes.

INTRODUCTION

Organophosphorus (OP) compounds are produced worldwide as chemical weapons and are a great threat to soldiers and civilians alike. They produce toxicity by inactivating AChE, the enzyme responsible for the breakdown of the neurotransmitter, acetylcholine. The resultant increase in the level of acetylcholine at cholinergic synapses, particularly in the brain and diaphragm produces an acute cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculation,

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behavioral incapacitation, muscular weakness, and convulsions culminating in death by respiratory failure. Current antidotal regimen for OP poisoning includes a combination of pretreatment with a spontaneously reactivating AChE inhibitor such as pyridostigmine bromide and postexposure therapy with anticholinergic drugs such as atropine sulfate and oximes such as 2-PAM chloride. These antidotal regimens were successful in preventing lethality of animals due to OP poisoning but were unable to prevent postexposure incapacitation, convulsions, performance deficits, or in many cases, permanent brain damage. These problems prompted the development of alternate protective measures capable of providing protection against the lethality of OP compounds as well as preventing postexposure incapacitation.

One approach is the use of enzymes, both stoichiometric and catalytic, as bioscavengers of highly toxic OP agents. Of these, plasma-derived Hu BChE and AChE have been recognized to be particularly effective *in vivo* (1). ChEs are closely related serine hydrolases that can be distinguished by their substrate specificity and inhibitor sensitivity (2). It was shown that ChEs irreversibly bind and inactivate OP toxic agents before they reach physiological targets. Exogenously administered ChEs protected animals from a variety of multiple LD50's of highly toxic OP without any toxic effects or performance decrements (3-6). Based on data from animal experiments, it was estimated that 200 mg of circulating BChE would be required to provide protection against $2XLD_{50}$ of soman (7). But the production of native Hu BChE in such quantities requires large quantities of plasma. In this study, we explored whether rHu BChE and rHu AChE can be substituted for native Hu ChEs as bioscavengers. Results suggest that rHu ChEs, even though were similar to native Hu ChEs in enzymatic properties, they differed greatly in their pharmacokinetic properties. Recombinant Hu ChEs possessed very short circulatory residence time in mice as compared to native Hu ChEs. In order to improve their circulatory residence times, we conjugated rHu ChEs with PEG-20,000 polymer and report here that the chemical modification of rHu ChEs with PEG greatly improved their circulatory stability in mice. However, when administered a second time, PEG conjugated rHu ChEs were cleared rapidly from circulation due to the production of neutralizing antibodies.

MATERIALS AND METHODS

Recombinant Hu BChE expression and purification: AdenoVator expression system (Q Biogene Inc, Carlsbad, CA) was used for the production recombinant Hu BChE. Hu BChE cDNA contains 6 X histidine tag at its 3' prime end for simplifying the purification of rHuBChE. Briefly, recombinant adenoviruses expressing Hu BChE (Ad-Hu BChE) and proline-rich attachment domain (Ad-PRAD) that was essential for producing tetrameric BChE were generated as described in the Q Biogene applications manual. These recombinant viruses were used for the production of fully tetrameric rHu BChE using human embryonic kidney epithelial cell line (293 A) as the host. 293A cells (10×10^6) were seeded in 150 cm^2 tissue culture dishes. After 24 h, the cells were simultaneously infected with 10 µl of 4^{th} cycle crude viral extract (CVE) of Ad-Hu BChE and 5 µl of 4^{th} cycle CVE of Ad-PRAD for 1 h using 10 ml of infection medium (DMEM containing 2 % fetal bovine serum (FBS), antibiotics penicillin and streptomycin, and sodium pyruvate) at 37^oC in the incubator. To the infected cells, 15 ml of growth medium (DMEM containing 10 % FBS, 50 µg/ml penicillin and streptomycin, and 50 µg/ml sodium pyruvate) was added and they were returned to the incubator for 7 to 8 days. Fifty to seventy culture dishes were infected in a single experiment. When BChE activity in the culture medium reached between 1.5-2.0 units/ ml, it was collected and subjected to centrifugation at 2500 rpm for 15 min at 4^oC to remove cells and debris. The clear culture medium was used for the purification of rHu BChE by ammonium sulfate fractionation followed by affinity chromatography using procainamide-Sepharose and nickel-affinity resin. Metal-affinity chromatography was necessary for the separation of rHu BChE from FBS AChE.

Recombinant Hu AChE expression and purification: Chinese hamster ovary cell line stably transfected with a full-length cDNA for Hu AChE was a generous gift from Dr. Oksana Lockridge (University of Nebraska, Omaha, NE). The cells were routinely grown in serum-free ultra culture medium containing 25 micromolar concentration of methionine sulfoximine**.** By the third or fourth day when the secreted AChE levels in the conditioned medium reached 80-150 units/ml, the medium was replaced with fresh culture medium. Recombinant Hu AChE in the conditioned medium was purified by a single step procedure involving procainamide-Sepharose affinity chromatography.

Sucrose gradient centrifugation: Aliquots of rHu BChE and rHu AChE (approximately 1 to 2 U) were mixed with catalase (11.3S, used as a sedimentation marker) and applied to linear 5-20% sucrose gradients prepared in 50 mM sodium phosphate, pH 8.0. The gradients were centrifuged at 75,000 X g for 18 h at 4^{o}C in SW41Ti rotor (Beckman Instruments, Fullerton, CA). Gradients were fractionated from top using an AutoDensiflow IIC (Buchler Instruments, Lenexa, KS), and fractions were assayed for BChE using the micro-Ellman assay (8).

Chemical modification of rHu ChEs with PEG: Ten to fifteen ml of rHu ChE (0.5 mg/ml) dissolved in 50 mM phosphate buffer pH 8.0 was added to a 25-fold excess (weight basis, 12.5 mg/ml) of succinimidyl-propionate–activated methoxy PEG-20,000 and the mixture was kept for 2 h at room temperature under gentle rotation. Following these conditions we generated homogeneous species of PEG modified native Hu BChE that was not affected by increasing the concentration of PEG to a 100-fold excess or the incubation time to 16 h. The resulting PEG-modified rHu ChEs were separated from free PEG by ultrafiltration using YM-30 membrane. The samples were freeze-dried and reconstituted in sterile saline (1500 to 2000 U/ml). The average molecular weight of PEG modified rHu ChEs was estimated by SDS-PAGE using 4-15% gradient gels.

Resistance to proteases: Native Hu BChE, rHu BChE, and PEG-rHu BChE **(**5 U/ml**)** were incubated with trypsin or chymotrypsin (1:10 enzyme to substrate ratio, Sigma Chemical Co, St. Louis, MO) in 50 mM phosphate buffer containing 0.05% bovine serum albumin at 370C for various time periods. BChE residual activity was measured using the Ellman assay (8).

Pharmacokinetics in mice: Eighteen mice (Balb/c, 8 weeks old, equal number of male and female, n=6) were subjected to one of the following treatments: (1) Hu BChE; (2) rHu BChE; (3) PEG-rHu BChE; (4) rHu AChE; and (5) PEG-rHu AChE. Each group received 100 unit of the protein by IP injections. Five µl of blood was drawn from the tail vein at various time intervals for the determination of BChE or AChE activity (8). The following pharmacokinetic parameters were determined from the time course curve of blood BChE or AChE concentration: mean retention time (MRT), maximal concentration (C_{max}), time to reach the maximal concentration (T_{max}), elimination half-life ($T_{1/2}$), and area under the plasma concentration time curve extrapolated to infinity (AUC), using a Windows-based program for non-compartmental analysis of pharmacokinetic data (9). Group means and standard deviations were calculated for all numerical data. Statistical evaluations were performed on pharmacokinetic parameters using the two-way ANOVA test. *P* value of less than 0.05 was considered significant.

Anti-ChE antibody assay: The presence of anti-ChE antibodies was followed by ELISA, using 0.2 U of rHu ChE per well as the plate-coating antigen (10). Mouse antibody binding to ChE was detected with peroxidase-labeled goat antibody to mouse IgG using ABTS substrate. Standard curves using purified mouse IgG were run with each assay to allow quantification of antibody response.

Other methods: Purity of rHu ChEs and their molecular size modification after conjugation with PEG was assessed by SDS-polyacrylamide gel electrophoresis (11). Determination of kinetic constants (*K*m, *K*ss) and inhibition constants (*K*i) for native and rHu ChEs and their PEG modified products were performed as described (8, 12). Protein concentration was determined using BCA protein assay kit (Pierce Co., Rockford, IL) using the enhanced method according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Characterization of rHu ChEs: Using a combination of protein purification techniques including ammonium sulfate fractionation, procainamide-Sepharose and nickel-affinity chromatographies for rHu BChE, and procainamide-Sepharose chromatography for rHu AChE, both rHu BChE and rHu AChE were purified to homogeneity. Figure 1A shows the electrophoretic mobility of purified rHu BChE and native Hu BChE by SDS-PAGE. Both rHu BChE (lane 1) and native Hu BChE (lane 2) demonstrated a similar migration pattern suggesting the molecular size of rHu BChE subunit was similar to that of the native protein. Similarly, rHu AChE migrated as a single band of 67 kDa, suggesting that it was a pure protein (panel B, lane 2). Collectively, these results suggest that full-length rHu ChEs were produced by this methodology.

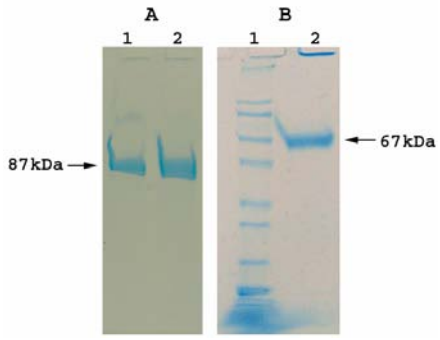

Figure 1: SDS-PAGE of rHu ChEs: Purified rHu BChE (5 µg, panel A, lane 1) and rHu AChE (3 µg, panel B, lane 2) were analyzed using 6 % or 4 to 15 % gradient polyacrylamide gels. Native Hu BChE (lane 2, panel A) was included for comparison. The molecular sizes for both rHu BChE and rHu AChE subunits were calculated to be 87 kDa and 67 kDa, respectively, by including molecular size standards (lane 1, panel B).

The catalytic parameters and inhibition constants for the purified rHu ChEs were compared with those for native ChEs by kinetic studies. These studies showed that *K*m, and *K*ss values for rHu BChE (Table 1) were not significantly different from those of native Hu BChE. Likewise, the inhibition constant for the classical active-site inhibitor ethopropazine (39 ± 25 nM) was similar to that for the native enzyme (48 ± 34 nM). The *K*m (89 ± 8 µM) and *K*ss (6.9 ± 0.6 mM) values for rHu AChE were also determined and found to be similar to reported values for native Hu AChE (13). These results suggest that the enzymatic properties of recombinant Hu ChEs were similar to those of the native enzymes.

TABLE 1: Kinetic parameters for native and recombinant Hu ChEs

ChE	*K*m (µM)	*K*ss (mM)
Hu BChE	12 ± 3	0.78 ± 0.12
rHu BChE	13 ± 3	0.79 ± 0.11
AChE*	90 ± 1	9 ± 2
rHu AChE	89 ± 8	7 ± 1

Values are mean SD for three independent experiments. * Values reported in literature (13).

The composition of the various molecular forms of rHu ChEs were determined by sucrose gradient centrifugation analysis. As shown in Figure 2, rHu BChE was mostly tetrameric in nature (> 95%). In contrast, rHu AChE was mostly monomeric and dimeric (70%) and less of tetrameric. As references, native Hu BChE and FBS AChE were included and they were fully tetrameric in form (Figure 2).

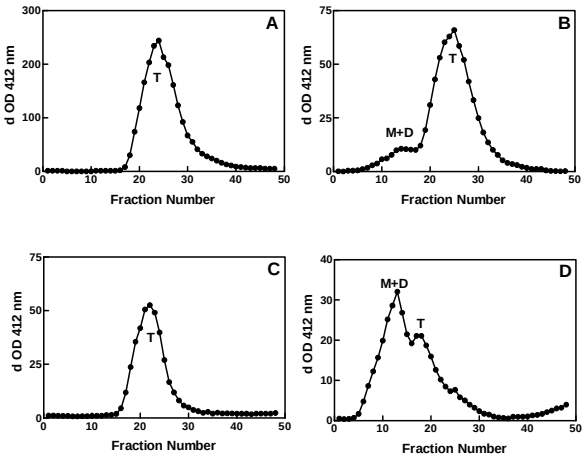

Figure 2: Sucrose gradient centrifugation analyses of rHu ChEs: Aliquots of rHu ChEs (1 to 2 U) were overlaid on linear 5-20% sucrose density gradients prepared in 50 mM sodium phosphate, pH 8.0 and centrifuged at 75,000 x g for 18 h at 4^{o}C. The gradients were fractionated from top and assayed for BChE and AChE activities as described (8). Panels (A-D) represent the following: A, native Hu BChE; B, rHu BChE; C, FBS AChE; and D, rHu AChE. M+D, monomer+dimmer; and T, tetramer.

Pharmacokinetic Behavior of rHu ChEs: The circulatory stabilities of rHu ChEs were compared with those of native ChEs, in mice. Time courses of native Hu BChE, rHu BChE, FBS AChE, and rHu AChE administered via i. p. injections were determined (Figure 3). Pharmacokinetic parameters (MRT, $T_{1/2}$, T_{max}, C_{max}, and AUC) were computed and shown in Table 2. Data in Figure 3 and Table 2 show that, unlike native Hu BChE and FBS AChE, rHu ChEs were cleared rapidly from the circulation of mice.

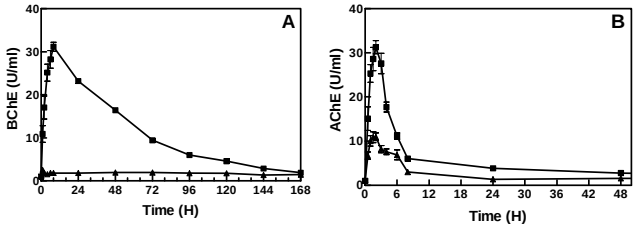

Figure 3: Time courses of rHu ChE (▲) and native ChE (■) in the blood of mice following a single injection of rHu ChE and native ChE, respectively. Circulatory profiles for BChE (A) and AChE (B) are shown.

Table 2: Pharmacokinetic parameters of rHu ChEs in mice.

Parameters	rHu BChE	Hu BChE	rHu AChE	FBS AChE
MRT (h)	N/D*	45.7 ± 1.7	6.9 ± 0.9	43.9 ± 3.1
$T_{1/2}$ (h)	N/D*	17.8 ± 0.3	1.6 ± 0.2	34.5 ± 2.8
T_{max} (h)	1	8	2	4
C_{max} (U/ml)	1.2 ± 0.2	30.3 ± 1.3	10.3 ± 1.26	27.9 ± 1.05
AUC (U)	80 ± 17	1768 ± 39	25 ± 8	1263 ± 105

* Values could not be determined as C_{max} is very close to baseline BChE activity.

PEG modification of rHu ChEs: To enhance the circulatory stability for rHu ChEs, they were chemically modified by conjugation with PEG-20,000 polymer. Figure 4 demonstrates the migration patterns of discrete bands of PEG-rHu BChE (panel A, lane 3) and PEG-rHu AChE (panel B, lane 3). Reaction of the 87 kDa rHu BChE and 67 kDa AChE subunits with PEG polymer resulted in the generation of PEG-rHu BChE and PEG-rHu AChE subunits with molecular sizes ranging from 120-200 kDa. Unmodified rHu ChEs were not found suggesting that PEG was conjugated to all most all of rHu ChE subunits.

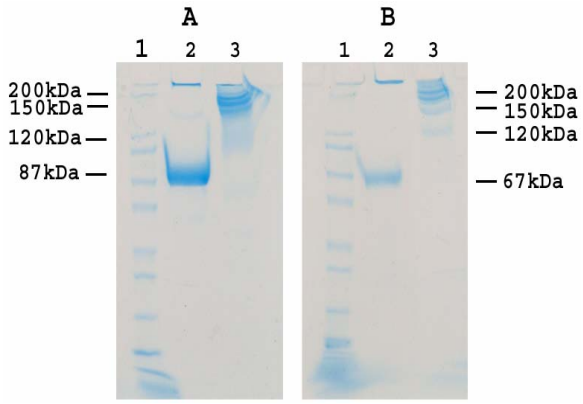

Figure 4: SDS-PAGE analyses of PEG modified rHu ChEs: Recombinant Hu BChE (panel A) and rHu AChE (panel B) were conjugated with PEG-20,000 as described under Methods and analyzed by SDS-PAGE using 4 to 15 % gradient gels. In both panels: lane 1, broad-range molecular size standards; lane 2, rHu ChEs before PEG conjugation; and lane 3, rHu ChEs after PEG conjugation. The migration rates of PEG-rHu ChEs were much slower than those of rHu ChEs due to PEG-conjugation.

Enzymatic properties of PEG-rHu ChEs: The effects of PEG modification on the catalytic properties of rHu ChEs were assessed by kinetic studies and compared with those for rHu ChEs. The data in Table 3 shows that the *K*m and *K*ss values for PEG-rHu ChEs were similar to those for rHu ChEs. These results suggest that PEG modification did not affect the enzymatic properties of rHu CHEs.

TABLE 3: Kinetic parameters for rHu ChEs and PEG-rHu ChEs

ChE	*K*m (µM)	*K*ss (mM)
rHu BChE	31 ± 3	1.25 ± 0.35
PEG-rHu BChE	48 ± 6	1.44 ± 0.35
rHu AChE	89 ± 8	6.85 ± 0.60
rHu AChE	85 ± 8	6.48 ± 0.60

Values are mean SD for three independent experiments.

Resistance to proteases: It was reported that PEGylation confers resistance to proteases and thereby prolongs the circulatory stability of proteins *in vivo* (14, 15). The effect of PEGylation on the susceptibility of rHu ChEs to trypsin and chymotrypsin was examined and the data was compared to that of native Hu ChE and rHu ChE. As shown in Figure 5, PEG-rHu BChE was more resistant to trypsin and chymotrypsin inactivation as compared to rHu BChE. In contrast, PEG modification of rHu AChE did not affect its resistance to chymotrypsin and trypsin (data not shown). Interestingly, native Hu BChE, when compared with rHu BChE, was more rapidly inactivated with trypsin but not with chymotrypsin. These results suggest that the proteolytic susceptibility of Hu BChE but not of Hu AChE was favorably modified by PEGylation.

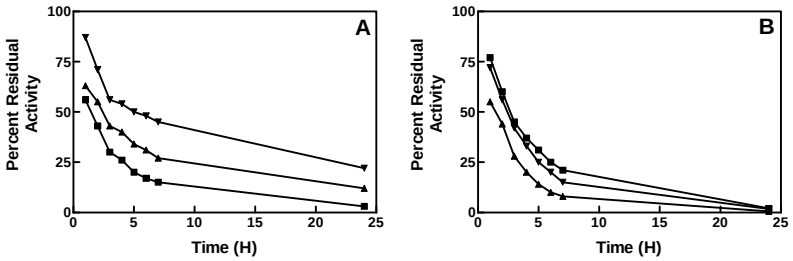

Figure 5: Protease susceptibility of PEG-rHu BChE. Susceptibility of PEG-rHu BChE (▼), rHu BChE (▲), and native Hu BChE (■) to trypsin (panel A) and chymotrypsin (panel B) are shown.

Circulatory stabilities of PEG-rHu ChEs in mice: The circulatory stabilities of PEG-rHu ChEs were examined in mice and compared with those of rHu ChE and native ChE. Time courses of 100 U of PEG-rHu ChEs, rHu ChEs, and native ChEs administered via i. p. injections were followed (Figure 6) and the pharmacokinetic parameters were determined. The data show that PEG conjugation resulted in a dramatic improvement in the circulatory stability of both rHu BChE and rHu AChE. For example, the MRT of 36 ± 2 h for PEG-rHu BChE was close to that observed for the native Hu BChE (45 ± 2 h). Similarly, the MRT of 51 ± 2 h for PEG-Hu AChE was significantly higher than that observed for rHu AChE (7 ± 1 h) and comparable to that for FBS AChE (44 ± 3 h).

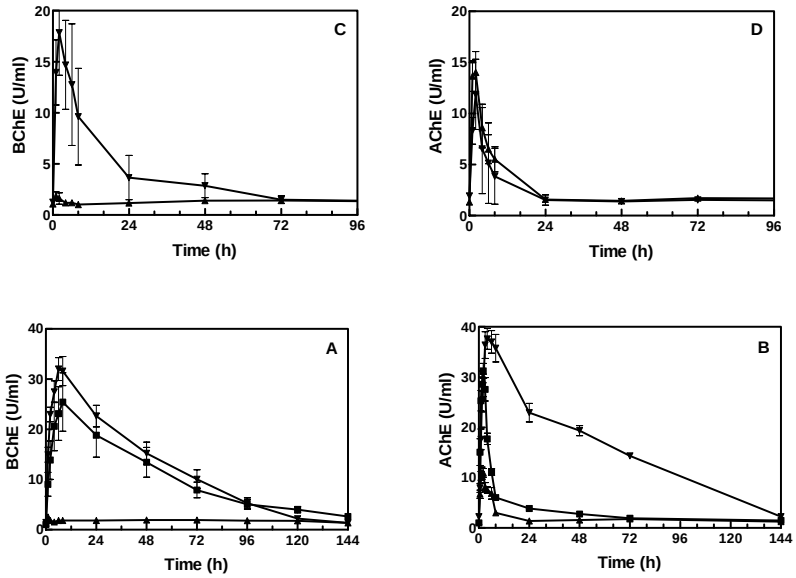

Figure 6: Time courses of PEG-rHu ChE (▼) and rHu ChE (▲) in the blood of mice following two injections of PEG-rHu ChE and rHu ChE, respectively. The circulatory profiles for the first injections of Hu BChE and Hu AChE are shown in panels A and B, respectively. The profiles for native ChEs (■) are also shown for comparison. The circulatory profiles for the second injections of Hu BChE and Hu AChE are shown in panels C and D, respectively.

Since multiple prophylactic treatments are envisioned necessary for maintaining long-lasting protective levels of circulating enzyme, mice were given a second injection of respective

enzymes (100 U) 28 days following the first injection, and their circulatory stability evaluated (Figure 6). Although the circulatory stability for the second injection of PEG-rHu BChE (MRT = 21.0 ± 10.0 h) was significantly larger than that for rHu BChE, it was shorter than that for the first injection (MRT of 36.2 ± 2.0 h). Similarly, the circulatory stability for the second injection of PEG-rHu AChE (MRT = 3.94 ± 2.0 h) was similar to that for rHu AChE (MRT = 7.95 ± 1.15 h), but much shorter than that for the first injection (MRT = 51.4 ± 2.1 h). These results suggest that, unlike the first injections of PEG-rHu ChEs, the second injections of PEG-rHu ChEs, were rapidly cleared from the circulation of mice.

Immunogenicity of PEG-rHu ChEs: Since PEG modification dramatically prolonged the circulatory stability of the first injection of PEG-rHu ChEs but not of the second enzyme injection administered 28 days following the first, the production of neutralizing antibodies against PEG-rHu ChE was investigated as a possible cause for the rapid clearance of the second injection. The sera of mice following two injections of rHu ChEs and PEG-rHu ChEs were examined for the presence of antibodies, by ELISA. As shown in Figure 7, no circulating anti-Hu BChE or anti-Hu AChE IgG were detected following the first injections of both PEG modified and unmodified rHu ChEs. In contrast, significant levels of antibodies to both unmodified and PEG modified rHu ChEs were detected following the second rHu ChE or PEG-rHu ChE injections. These results suggest that the poor circulatory stability observed for the second injection of PEG-rHu ChEs was due to the production of neutralizing anti-ChE IgG in mice.

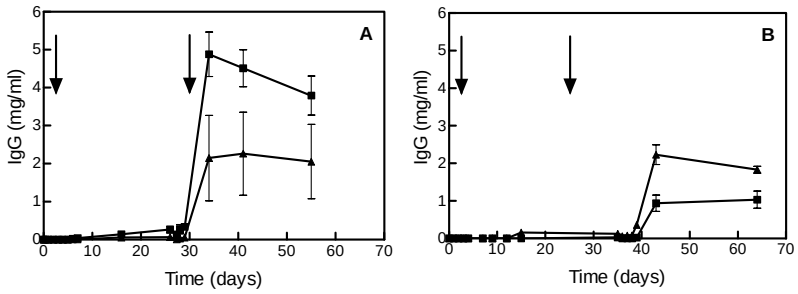

Figure 7: Antibody levels in sera of mice following two injections of rHu ChEs (■) and PEG-rHu ChEs (▲). Arrows indicate time of first (on day 1) and second (on day 29) injections. Panels A and B represent the antibody profiles for BChE and AChE, respectively.

PEGylation of rHu BChE reduced its immunogenicity by almost 50 %, suggesting that some but not all epitopes responsible for the induction of anti-enzyme antibodies were masked by the PEG polymer. These results are consistent with the increased circulatory stability of the second injection of PEG-rHu BChE compared to rHu BChE. In contrast, PEGylation of rHu AChE appeared to increase the immunogenicity of rHu AChE, which could account for the much rapid clearance of the second injection of PEG-rHu AChE compared to the first injection.

CONCLUSIONS

This study describes the expression and purification of rHu BChE and rHu AChE and a comparison of their biochemical and pharmacokinetic properties with those from plasma-derived enzymes. Results suggest that rHu ChEs are not different from ChEs from native sources in

molecular size and catalytic properties but differ significantly with regard to circulatory stability in mice. Native Hu BChE and FBS AChE remained in the circulation of mice for long periods of time where as rHu ChEs cleared rapidly from circulation. Conjugation of rHu ChEs with PEG-20,000 polymer resulted in the formation of higher molecular size rHu ChEs but this modification did not affect their catalytic properties. When administered into mice, a significant improvement in the circulatory stability was observed for PEG-rHu ChEs. Pharmacokinetic parameters for PEG-rHu ChEs were similar to those for native ChEs. However, a second injection of PEG-rHu ChEs into mice given 28 days later, showed a much rapid rate of clearance as compared to the first injection. The second injection of PEG-rHu BChE was much more stable as compared to rHu BChE and PEG-rHu AChE. The significant differences observed in the clearance profiles of the first and second injections of PEG-rHu ChEs coincided with induction of anti-enzyme immunity in mice. Anti-ChE IgG levels were undetectable in the sera of mice following the first injection of PEG-rHu ChEs but these levels increased significantly following the second injection. Therefore, antibody mediated clearance could account for the rapid clearance rates observed for the second injections of PEG-rHu ChEs. In conclusion, although PEGylation represents a suitable simple strategy to improve the circulatory stability of rHu ChEs, it may not be a suitable approach to eliminate the immunogenicity of these enzymes.

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